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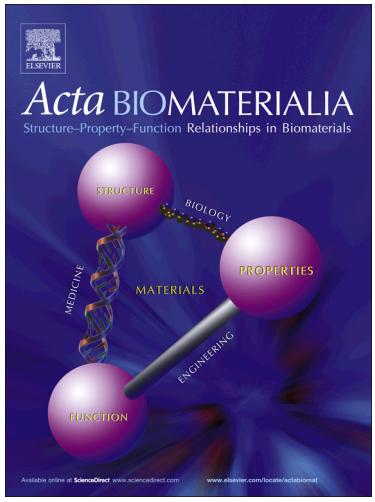

Full length article

Electroneutral polymersomes for combined cancer chemotherapy

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Title

Electroneutral polymersomes for combined cancer chemotherapy

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Abstract

Combination cancer chemotherapy provides an important treatment tool, both as an adjuvant and neoadjuvant treatment, this shift in focus from mono to combination therapies has led to increased interest in drug delivery systems (DDS). DDSs, such as polymersomes, are capable of encapsulating large amounts of multiple drugs with both hydrophilic and hydrophobic properties simultaneously, as well as offering a mechanism to combat multi drug resistant cancers and poor patient tolerance of the cytotoxic compounds utilised. In this article, we report the formulation and evaluation of a novel electroneutral polymersome capable of high encapsulation efficacies for multiple drugs (Doxorubicin, 5-Fluorouracil and leucovorin). The *in-vivo* biodistribution of the polymersome were established and they were found to accumulate largely in tumour tissue. Polymersome encapsulating the three chemotherapeutic drugs were assessed both *in-vitro* (BxPC-3 cell line) and *in-vivo* (following intratumoral and intravenous administration) and compared with the same concentration of the three drugs in solution. We report better efficacy and higher maximum tolerated dose for our combination drug loaded polymersomes in all experiments. Furthermore, intratumorally injected combination drug loaded polymersomes exhibited a 62% reduction in tumour volume after 13 days when compared with the free combination solutions. A smaller differential of 13% was observed for when treatment was

administered intravenously however, importantly less cardiotoxicity was displayed from the polymersomal DDS. In this study, expression of a number of survival-relevant genes in tumours treated with the free chemotherapy combination was compared with expression of those genes in tumours treated with the polymersomes harbouring those drugs and the significance of findings is discussed.

Key words

Combination chemotherapy, polymersome, pancreatic cancer, nanoparticle, drug delivery system

1.0 Introduction

The advantages offered by combining two or more chemotherapies has been recognised and documented from as early as the mid twentieth century [1]. It is now widely accepted that most cancers respond better to combination drug therapy rather than single agent therapy. The accessibility of combination therapies has benefitted from the development of novel formulations such as nanoparticle (NP) drug delivery systems (DDSs). The first commercially available DDS was that of Doxil® in 1996, a liposome containing the chemotherapeutic agent doxorubicin (Dox) [2,3]. Liposomes have been investigated for the delivery of many anticancer agents such as anthracyclines[4-6], platinum compounds[7], antimetabolites[8] and vinca alkaloids[9-11] aiming to reduce their side effects without affecting their efficacy. Liposomes take advantage of the enhanced permeability and retention (EPR) effect of the tumour vasculature [12,13]. Co-encapsulation of multiple drugs in liposomes has shown to provide a synergistic action increasing their efficacy [14-16]. Although there is much evidence to suggest that liposomes have had a significant impact on existing chemotherapeutic treatments, their drawbacks include low stability and shelf life and diffusion of the drug across the liposomal membrane with time [17]. The limitations of liposomal delivery systems have led to a shift in focus towards more stable alternatives. Discher *et al.* [18] demonstrated in the late 1990s, the ability of diblock copolymers to assemble into bilayer vesicles termed polymersomes [18,19]. Polymersomes provide an alternative DDS by combining the advantages of bilayer forming liposomes with enhanced stability and shelf life [20,21]. The polymer backbone can be designed to include desirable properties such as biodegradability

and biocompatibility [22,23]. The large molecular weights of polymers used for these structures can significantly improve their membrane properties such as permeability, thickness and robustness [24]. Similar to liposomes, polymersomes have the ability to encapsulate both hydrophilic and hydrophobic compounds, however owing to the larger size of the hydrophilic cores [25] and thicker hydrophobic bilayer [26], the amount of encapsulated drug is increased. Recently there have been some interesting examples of polymersomes encapsulating multiple anticancer drugs [27-29]. Dox and paclitaxel loaded polymersomes have shown enhanced anticancer activity against head and neck squamous cancer cells [30] whereas siRNA co-encapsulated with Dox in block copolymer polymersomes in human gastric cancer cell lines has shown increased efficacy at lower doses [31]. There are further examples of combination anticancer therapy using 5-Fluorouracil (5-FU) and Dox loaded in NPs such as dendritic nanomicelles [32], polymer drug conjugated NPs [33] and nanocomplexes [34] all displaying a synergistic action against different types of cancer [35]. Thus, NP DDSs are proving to be a valuable tool for the co-delivery of multi drugs, they are not only effective in treating resistant and recurrent tumours but also have the potential to reduce the time restraints of current combination therapies which are often delivered over a number of consecutive days.

In this manuscript, we describe the simultaneous encapsulation of three anticancer agents, Dox, 5-FU and leucovorin (LV), into a random copolymer polymersome. We report excellent encapsulation efficiencies with both single and multiple occupancy within the polymersomes with the hydrodynamic radius suitable for endocytosis. The *in-vitro* analysis suggests that the polymersome formulation significantly enhances the efficacy observed with the free drug combination therapy. We describe, for the first time, the biodistribution of this particular DDS *in-vivo* using fluorescence imaging to show enhanced tumour accumulation. The therapeutic efficacy of the loaded polymersomes was examined using ectopic pancreatic BxPC-3 tumours in mice after both intratumoral and intravenous injection where we report a reduction in tumour growth when compared with treatments using the free solutions. Finally, the treated tumours were analysed for the expression of forty relevant genes. Treatment with the combination drug loaded polymersomes caused a greater reduction in pro- survival gene expression in lesions when compared with expression of those genes following

treatment with the free drug to combination and the implications of these findings are discussed

2.0 Materials and Methods

Materials

DOX-HCl, 5-FU, LV calcium, indocyanine green (ICG), dialysis membrane (MWCO 12-14kDa), PBS tablets pH 7.2 and chloroform were obtained from Sigma-Aldrich, UK. RPMI 1640, FBS, pen/strep were sourced from Thermofisher Scientific, UK. Matrigel® basement matrix was acquired from Corning Inc.

2.1 Preparation and characterisation of polymersomes

Polymersomes were prepared from a previously described random co-polymer [36] containing polyethylene glycol (PEG, Mn 500Da), cholesteryl and a decyl chain in a 73:12:15 %w/w ratio. Synthesis of the polymer is well documented with characterisation carried out using GPC (Agilent MDS-THF, data collected by Agilent GPC/SEC Software Version 1.2.3182.29519). Formulation was achieved using the reverse phase evaporation method; 0.5mL polymer in chloroform (5mg mL^{-1}) was evaporated to dryness under reduced pressure in a round bottom flask to form a thin film. Depending on the application an appropriate volume and concentrations of Dox, 5-FU and LV were added on top of the film and again evaporated to dryness under the same conditions. 1mL Chloroform was added to the round bottom flask and sonicated for 15 minutes using a Branson 3510 bath sonicator (230V) at room temperature, after which 0.5mL of the polymer in PBS (5mg mL^{-1}) and 0.5mL PBS were added and the resulting mixture sonicated for a further 30 minutes forming an emulsion. The chloroform layer was then under reduced pressure at 45℃. The resulting suspension was freeze dried and resuspended as per requirement. Particle size and zeta potential of polymersomes were determined using Malvern Nano-ZS Zetasizer without the use of any size extrusion techniques. SEM images were carried out by applying a small amount of the polymersome onto an aluminium stub. The sample was then lyophilised overnight before being sputter coated with gold and palladium. The images were recorded using a FEI Quanta SEM under a high vacuum in secondary electron mode.

2.2. Encapsulation efficiency and release studies of combination drugs

Encapsulation efficiency of the polymersomes were evaluated by centrifugal filtration using a dialysis membrane and centrifuging the polymersomes at 4°C at 3000rcf for 2 hours. The polymersomes were prepared as described in section 2.1, following the creation of a thin polymer film, 100 µL Dox (0.116 mg mL^{-1} PBS), 100 µL 5-FU (5.2 mgs mL^{-1} PBS) and 100 µL LV (25.6 mgs mL^{-1} PBS) was added and the solution evaporated to dryness with the chloroform added as above for the combination solutions within the polymersome. The freeze dried sample was reconstituted in 1 mL PBS (containing 5 mg mL^{-1} polymer, 11.6 µg mL^{-1} Dox, 0.52 mg mL^{-1} 5-FU and 2.56 mg mL^{-1} LV), the individual polymersomal formulations were prepared to contain an identical amount of one component (only one from: 11.6 µg mL^{-1} Dox, 0.52 mg mL^{-1} 5-FU or 2.56 mg mL^{-1} LV) alongside 5 mg mL^{-1} polymer. 0.5 mL of each of the polymersome suspensions were added to a semipermeable membrane (MWCO 12-14kDa), secured at both ends and suspended within a centrifuge tube before centrifugation. The resultant filtrate was collected and analysed for unencapsulated drug. Dox was analysed by fluorescence spectroscopy at Ex 485nm/Em 580nm ($y = 91.324x$, $R^2 = 0.9881$), 5-FU and LV by UV spectroscopy at absorbance maximum 265nm($y = 0.0581x$, $R^2 = 0.9992$) and 285nm ($y = 0.0543x$, $R^2 = 0.9998$) respectively. *In vitro* release studies of polymersomes were conducted after removing the unencapsulated drug/s and keeping the polymersomes in the sealed dialysis membrane. The sealed semi permeable membrane was stirred in 10 mL PBS maintained at 37°C, samples were removed at fixed intervals of time and analysed for individual drug concentration, the same volume of PBS was replaced after each removal to maintain sink conditions.

2.3. *In-vitro* study to establish efficacy

BxPC-3 cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% pen/strep. 100 µl, $1x10^5$ cells mL^{-1} were seeded into each well on 96 well plates and allowed to adhere overnight. Polymersome solutions were prepared as described for the encapsulation efficiency and release studies, and sterile filtered with a 0.45µm Millex MCE syringe filter. 100µl of either free solution of individual drugs at identical concentrations, combination free solutions or combination drugs encapsulated in polymersomes were applied to cells generating a 1:1 dilution of test solution with the

cell media, giving final cell concentrations of; 5.8 µg mL^{-1} Dox, 0.26 mg mL^{-1} 5-FU or 1.28 mg mL^{-1} LV and 2.5mg mL^{-1} polymer. The cells were then incubated at 37℃ (20% O_2 5% CO_2) for 22 hrs. An MTT assay was then performed by incubating each well with 20µl of MTT solution (5mg/ml in PBS) and 100 µl of fresh medium for 3 hours and reading the absorbance in DMSO at 570nm using a Fluostar Omega microplate reader.

2.4. *In-vivo* biodistribution assay of polymersomes

In these studies, all animals were treated humanely and in accordance with licenced procedures under the UK Animals (Scientific Procedures) Act, 1986. Animals were anaesthetised using 150µl intraperitoneal injection of water for injection: hypnorm: hypnovel (2:1:1) (VetaPharma Ltd., U.K.). 50µl polymersomes (5mg mL^{-1}) loaded with 0.2mg mL^{-1} ICG were prepared as previously described with 100 µL of 2mg mL^{-1} ICG replacing the Dox/5-FU/LV and sterile filtered with a 0.45µm Millex MCE syringe filter before being injected into the tail vein of mice previously implanted with ectopic BxPC-3 tumours as described in section 2.5. Following administration, animals were placed in Xenogen IVIS® Lumina imaging system chamber maintained at 37°C equipped with an ICG filter set (Ex 705–780 nm; Em 810–885 nm). Images were recorded at regular intervals up to 22 hours to observe the *in-vivo* biodistribution of the DDS in real time. After 22 hours, the mice were sacrificed and their organs and tumours were harvested and imaged for the presence of ICG fluorescence. All data were analysed using Living Image® software version 2.60 and reported relative to the background emission.

2.5. Treatment efficacy after Intratumoral and intraveneous injection

BxPC-3 cells ($5x10^6$ cells per mouse) were resuspended in RPMI 1640 medium and Matrigel® in 1:1 ratio and implanted subcutaneously into the rear dorsum of NOD-SCID mice (NOD.CB17-Prkdcscid/NCrHsd). Tumour formation occurred approximately 2 weeks after tumour cell implantation, tumours were measured via calipers and tumour volume calculated using the formula (LxWxH)/2). Once tumours reached 200-250mm^3 animals were grouped and treatment commenced.

After the ectopic tumours reached the desired volume, the mice were randomly segregated into 6 groups of 3 or 4 mice in each group. Prior to treatment animals receiving the intratumoral injections were anaesthetised using intraperitoneal

administration of Hypnorm/Hypnovel. Treatment groups consisting of; control untreated animals; those treated with empty polymersomes (intratumoral), combination solution (intratumoral), combination loaded polymersomes (intratumoral), combination solution (I36V) and combination loaded polymersomes (IV). The concentration of 5-FU and LV to be added was calculated using the therapeutic range in humans (450-600 mg/m^2 and 20 – 100 mg/m^2 respectively)(Table S1). Due to the extremely recalcitrant nature of pancreatic cancer the concentration for Dox was higher than the therapeutic range (generally 40 – 75 mg/m^2) but in line with similar studies [37]. Calculations were based on an average surface area of an adult human body being 1.73m^2 with an average weight of 60 kg. Calculations were originally based on an estimated average mouse weight of 20g and adjusted to allow for the actual average weight of the mice used of 23.5g. Solutions of 100µl Dox (5mg mL^{-1} in water), 250µl 5-FU (8mg mL^{-1} in water) and 50µl LV (5mg mL^{-1} in water) were added to the polymer thin film and evaporated as before. The final freeze dried sample was resuspended in 50 µL PBS to generate the required concentrations and sterile filtered with a 0.45µm Millex MCE syringe filter. Polymersomes (polymer (42.5mg/kg) loaded with Dox (4.3mg/kg), 5-FU (17mg/kg) and LV (2.1mg/kg)) were injected either intratumorally or intravenously at 100µl volume on Day 0 and Day 5 of treatment in mice according to their respective groups whereas the untreated group received no treatment. Tumour volume and body weight was measured every day for 13 days after intratumoral injection and 7 days after intravenous injection. On completion of data collection, mice were sacrificed and their tumours and hearts were harvested. Hearts were weighted and stored in formalin free tissue fixative for further observations. Tumour doubling time was calculated using an online calculator [38].

2.6 Assessment of Cardiotoxicity after intravenous treatment

Hearts from the above experiment were weighted and histologically stained in order to assess cardiotoxicity. Fixed hearts were dehydrated in a series of ascending concentrations of methanol; 50%, 70%, 90% and 100% (one hour each).Tissue samples were washed twice in 100% methanol (one hour each). Samples were cleared with a xylene substitute (Sigma, UK) for 4 hours and embedded in molten paraffin wax (Sigma, UK). A microtome (RM2135; Leica, Germany) was used to section heart tissue to 5µm thickness. Sections were placed on glass slides and dried overnight before being hematoxylin-eosin stained (H & E).

2.7 Gene expression analysis on xenografts treated with combination polymersomes

To establish the effect of drug loaded polymersomes on the expression of specific genes involved in the development and progression of pancreatic cancer. 40 candidate genes involved in pancreatic cancer progression were analysed with 28 of these having a Cp value of less than 40 (BID, BAX, BCL2, BCL2L1, BAD, BIK, EPCAM, MET, ALDH1A1, VEGFA, VEGFC, NME1, CA9, CTGF, RUNX2, CNDP2, TFE3, ARL8B, CLN3, TOP2A, HIF1A, ABCC1, AGA, CLCN7, CASP3, IL8, NANOG and SOX2). Tumours in mice from untreated, combination solution (IV) and combination polymersomes (IV) were excised at the experimental endpoint (Day 7). Tumours were snap-frozen using liquid nitrogen and RNA was extracted using Trizol reagent (Life Technologies) in accordance with the manufacturer's instructions [39]. 1.25 µg of RNA per tumour was reverse transcribed using the First Strand cDNA synthesis kit (Roche) in accordance with the manufacturer's instructions [40] and the cDNA from the tumours in each group were pooled. Samples were analysed in duplicate on Roche RealTime Ready custom 96 well gene panels (Roche) containing the relevant target genes. The results were normalised to a reference gene set (Beta actin, 18s ribosomal RNA and GAPDH) and the fold changes in gene expression in tumours treated with combination solutions and with combination polymersomes were compared to untreated expression levels; genes considered up-regulated (≥2) or down-regulated (≤0.5) and those which satisfied a p value <0.05 (to the left of the vertical green line, Figure 13A) were deemed statistically significant (student t-test). Results shown are mean ± SE of ≥3 pooled tumours analysed in duplicate.

2.8 Statistical analysis

All data are reported as n=3 unless otherwise stated. Error bars indicate ±SEM. Statistical significance of groups was determined using Unpaired Student's t test in Graphpad Prism Version 5.01.

3.0 Results and Discussion

3.1 Characterisation and evaluation of polymersomes

The amphiphilic random copolymer used for the preparation of polymersomes was composed of three components, two hydrophobic components comprising of a decyl chain, and cholesterol as well as a hydrophilic component, poly (ethylene) glycol (PEG) with an average molecular weight of 500Da. Polymerisation was achieved by free radical polymerisation using AICN as the initiator. The mass of the polymer was determined by GPC and found to have a Mn of 3.2KDa with a PDI of 3.6. As the polymer formed was a random co-polymer, the exact positioning of each monomer relative to each other is not guaranteed. However, on formation of the polymersome, due to the amphiphilic nature of the components the PEG 500 forms the hydrophilic inner core and outer corona of the polymersome whereas the cholesterol and decyl chain form the hydrophobic bilayer of the polymersomes as depicted in Figure 1A.

Multiple drug loaded polymersomes were prepared by the reverse phase evaporation method as described above in section 2.1 with each of the three drugs encapsulated into the aqueous core. The hydrodynamic radius, PDI, zeta potential and encapsulation efficiencies of the polymersome formulations can see seen in Table 1. Figure 1B displays the SEM images of the Dox loaded polymersomes, the spherical morphology is apparent with the NP's of a similar size range. The size of all polymersomes was found to be less than 300 nm with a PDI of less than 0.5 and low zeta potential, all of which are expected for electroneutral NPs of this type. The simultaneous encapsulation of 5-FU, Dox and LV eliminates their solution incompatibilities and allows their simultaneous administration with enhanced therapeutic effect. The encapsulation efficiency of all the compounds was greater than 70 % (Table 1) and in agreement with similar studies reported in the literature [41,42].

In-vitro release studies indicate that polymersomes demonstrate a burst effect followed by a fast release of drugs (Figure 2) with more than 65% of 5-FU released within 8 hours, whereas just over 40% for Dox and 30% LV was released after 8 hours following the initiation of the study. Almost all of 5-FU was released after 24 hours however, release of Dox and LV remained constant. Hence, even though the release of Dox and LV was fast initially, it remained steady to roughly less than 50% over 24 hours. It was concluded that the amphiphilic copolymer polymersomes were electroneutral, of appropriate size within the nano range required for endocytosis, with PDI's less than 0.5 indicative of sufficiently monodispersed systems, display good

encapsulation of multiple drugs and are capable of releasing their encapsulated cargo into the surrounding environment.

3.2. Cell viability studies

To evaluate the synergistic action of the combination drugs on the cell viability of BxPC-3 cells, free solutions of 5-FU, LV and Dox were compared to their combination free solution and combination drugs loaded in polymersomes at concentrations 5-FU (0.26 mg mL^{-1}) , LV (1.28 mg mL^{-1}) and Dox (5.8 µg mL^{-1}) and 2.5mg mL^{-1} polymer. As seen in Figure 3, the viability of individual drug solution treated cells were 91.7±11.0% for Dox, 91.3±8.6% for 5-FU and 107.7±7.0% for LV indicating only limited toxicity at the above mentioned concentrations. When the same cells were subjected to the combination solution toxicity was higher with 74.9±6.6% viable cells, showing a synergistic action with approx. 25% cytotoxicity. The toxicity was greatly enhanced when the same concentrations were combined within polymersomes, they displayed ~65% cell toxicity with only 36.5±3.6% viable cells. Furthermore, polymersomes with single/dual occupancy drugs were prepared with identical concentrations and the cell viability established. In each of the polymersomes assessed an enhanced toxicity was observed when compared to non polymersomal solutions (Fig. S1). The highest toxicity was recorded from the 5-FU/LV combination of 49.5% which was marginally more toxic than the 5-FU single occupancy polymersome (53% cell viability). This increased toxicity provided by the polymersome encapsulated drugs could suggest enhanced uptake of the drugs. Since the toxicity of the combined drugs in the polymersomes was greater than that exhibited by the individual components of the formulation, the data suggested that a synergistic effect may be occurring. This could provide significant and unforeseen benefits in using such an approach in cancer therapy. To compliment this research, a further polymersome was prepared to contain a higher (x2) concentration of Dox with all other parameters remaining unchanged. The cell viability study (Fig. S2) shows a less than 20% cell viability with the combination PS Dox x2, compared with a 48% cell viability achieved from the same concentration of free solutions. This data would suggest that a. the loading potential of the PS system has not yet been met and b. the synergistic effect could be optimised further, thus creating a more potent drug cocktail. However, this higher concentration

of Dox was not used in the *in-vivo* study as cardiotoxicity was of utmost concern, in particular with the free solution controls.

3.3 In-vivo biodistribution of polymersomes

In order to ascertain the behaviour of these polymersomes *in-vivo* we employed polymersomes loaded with Indocyanine green (ICG). ICG is a near infrared dye, commonly used for *in-vivo* imaging because of its water solubility, easy loading into NPs, good tissue penetration and reduced light scattering [43,44]. Although the method is semi quantitative, it provides the advantage of being able to monitor the behaviour of the polymersomes in the body in real time. Polymersomes loaded with ICG (0.2mg/mL) were injected into the tail vein of mice bearing ectopic BxPC-3 tumour. As seen from 4A, the fluorescence after initial injection shows that polymersomes containing the fluorophore were present in the tumour . The polymersomes in peripheral circulation (as indicated by the signal from the extremities as a surrogate fluorescent signal for circulatory vehicle) slowly started to dissipate with a significant amount retained in the tumour tissue as observed 22 hours following administration. Whilst the initial signal from the tumour may be attributed to both circulating and retained vehicle, a plot of tumour fluorescence expressed relative to peripheral tissue fluorescence (Figure 4B) suggests a 6-fold degree of accumulation of polymersomes in the tumour after 8 hours as compared to systemic circulation. Although the signal disappeared from the peripheral tissues with time, the relative amount in the tumour only decreased slightly after 22 hours post injection. To further confirm the accumulation of polymersomes in tumour tissue and determine their tissue distribution, the mouse was dissected, and individual organs were collected for fluorescence observation. Figure 5 shows the individual excised organs with the fluorescence emission from each organ quantified and compared to a blank. The results suggested that there was a significant degree of polymersome accumulation in the tumour, however a significant amount was also seen in the liver. These data support our suggestion that the fluorescent signal from circulating vehicle was decreasing more rapidly than that in the tumour since the fluorescent signal from more highly perfused organs such as the lung and kidney were lower than the less perfused tumour tissues. Figure 5 (insert) displays the fluorescence emission observed from the faeces of the mouse before and after injection with polymersome containing ICG,

further confirming excretion through the liver. This suggested biodistribution agrees with previous studies carried out by Photos *et al.* and Fei Lu *et al* [45,46]. They demonstrated that PEG based polymersomes were mainly eliminated by the liver followed by the spleen with a small amount being eliminated by the kidneys. In addition, the lack of accumulation in the heart was an encouraging indication for the potential use of NPs with cardio toxic compounds such as Dox.

3.4 In-vivo therapeutic efficacy following Intratumoral injection

Intratumoral injection has been shown to improve tumour concentrations and tumour-organ ratios of injected drugs [47]. Our rationale for using it here was to preclude any unforeseen pharmacokinetic complications and primarily examine the potential therapeutic efficacy of our preparation. NOD-SCID mice were implanted with BxPC-3 tumours and the relevant formulation was administered intratumorally when the tumour size was appropriate. On Day 0 and Day 5 mice were injected with either the combination drug loaded polymersomes (comb PS) or combination of three drugs in free solution (comb soln) and their impact on tumour growth was compared with that in mice injected with blank polymersomes and those that received no treatment. Tumour volume was measured each day until the end of treatment (Day 13).

Throughout the duration of the study, the body weight of the mice was regularly recorded and found to remain steady at 90 ± 10% (Figure 6A) indicative of limited toxicity caused by the Intratumoral administration of the chemotherapeutic drugs. The individual growth pattern of the tumours is illustrated in Figure 6B for each group. It was interesting to note that there was a spiked increase in recorded tumour volume 2 days after intratumoral injections for both groups containing the therapeutic agents. The injections were carried out on Days 0 and 5, the increase is then seen in Days 1 and 2 and consecutively on Days 6 and 7 for the combination solutions and the combination drug loaded polymersome groups. The increase in recorded volume for the combination solution treated group is more prominent (30-40%) than those treated with the combination drug loaded polymersomes (5-10%). This increase in recorded volume is most likely due to the vesicant nature of the Dox [48] and or the inflammatory nature of 5-FU [49], causing local inflammation of the surrounding tissues at the site of injection. This is further confirmed by the lack of any such increase observed with the free polymersome suspension of identical volume. The reason for the reduced

immune response observed in the combination drug loaded polymersome may be attributed to the protection offered by the polymersome bilayer inhibiting Dox and, to some extent 5-FU, from causing such an inflammatory immune response. In addition, the particulate nature of NPs allows them to diffuse through the interstitial space and lymphatic vessels of the tumour leading to a more homogeneous distribution throughout the tumour tissue, causing a less pronounced inflammatory effect.

Figure 7 displays a photograph of each group containing both the mouse and the excised tumour. It is evident that the smallest tumour was excised following polymersomal treatment with the combination drugs (comb PS), the next most effective treatment was from the free solutions (comb soln) with both the control and the blank polymersomes displaying the largest tumours after 13 days. Figure 8A shows the percentage increase in tumour weight of all the groups at the end of the study. The group treated with blank polymersomes had no effect on the tumour toxicity showing 160.2±21.6% increase in tumour weight from the initial and having the same trend as the untreated group whose tumour weight increased by 173.5±42.0% after 13 days. It was anticipated that the free drug solutions would provide good reduction in tumour weight since the concentrations of Dox, 5-FU and LV were at therapeutic doses. Indeed, the group treated with the combination solution of free drugs exhibited a significant reduction in tumour growth with an increase of only 59.6±32.8% tumour weight from the start of the experiment. However, the group treated with combination drug loaded polymersomes had the maximum cytotoxic effect on the tumour showing a decrease of 2.3±20.4% in the tumour weight after 13 days which is significantly better than the same concentration of free drug solution.

Tumour doubling times were calculated to be 10.3±1.20 and 10.0±1.4 days for the control group and the blank PS, respectively. In contrast, the rate of tumour growth for the combination solutions was calculated to be 18.0±5.0 days whereas the combination drug loaded polymersome therapy displayed a tumour doubling time calculated to be almost four times slower than the combination solutions at 67.0±22.0 days (Figure 8B).

3.5 In-vivo toxicity after Intravenous injection

The observation of enhanced retention of polymersomes in tumour tissue of ICG loaded polymersomes, provided the basis for the exploration of antitumor activity of

multiple drugs-loaded polymersomes administered by intravenous injection. Again, mice with ectopic BxPC-3 tumours were divided randomly into groups receiving intravenous injection of combination free drugs solution (comb soln) on Day 0 and Day 5, a group receiving combination drug loaded polymersomes (comb PS) at the same concentration on Day 0 and Day 5 and finally a control group that received no treatment. As with the previous study, the body weights of all mice were recorded on a regular basis and data are shown in Figure 9A. The mice treated with combination free drug solution (comb soln) displayed a decrease in the body weight from the first day of treatment which potentiated after the second treatment and the weight of mice fell to 80.0 ± 4.01% from their initial weight indicating accumulated systemic toxicity and leading to the termination of the study on day 7. The mice were weary, distressed and unable to eat and this further exacerbated weight loss. The mice in the untreated and combination drug loaded polymersomes group (comb PS) were relatively healthy with only a 10% loss in body weight of the latter towards the end of the experiment. The rapid weight loss of mice treated with combination free drug solution is indicative of acute cardiotoxicity of Dox at the concentration administered in mice. Dox has been reported to exhibit acute cardiomyopathy which is dose dependant leading to congestive heart failure at higher doses [50-52]. Other effects of anticancer drugs such as 5-FU and Dox include nausea, vomiting, poor appetite, alopecia and hematopoietic suppression [53,54]. Although cardiotoxicity of Dox remains the most feared side effect of chemotherapy, the encapsulation of Dox into liposomes and other NPs has helped reduce its cardiotoxicity, the most common example being the marketed pegylated liposomal Dox, Doxil® [55-57]. The encapsulation of the combination drugs into polymersomes at the same concentration produced limited toxic side effects in the mice indicating a higher maximum tolerated dose. Figure 9B displays the % tumour growth of each group. A steady increase in tumour volume was observed for all three groups throughout the 7 days of treatment, with the tumour volume increasing to 74.8% ± 11.8% in the control group and an increase of 53.7 ± 11.2% in the group treated with the combination free drug. Mice treated with combination drug loaded polymersomes displayed the lowest increase in tumour volume of 40.1% ± 10.8% from initial volume. Following the premature end of the experiment, all the mice were euthanized, and their tumours and hearts were excised. Figure 10 displays a photograph of one mouse from each group with the corresponding excised tumour. This image reinforces the data from Figure 9B and displays the smallest tumour

excised being that from the combination drug loaded polymersomal treatment group. Although the degree of tumour inhibition afforded in these experiments was not as dramatic as that observed when the combination drug-loaded polymersomes were administered intratumorally, we believe that these data demonstrate significant therapeutic benefit over that observed with the free drug, particularly in terms of reducing adverse effects. It should further be noted that although preliminary in nature, the data trend, in terms of tumour size reduction following intravenous administration certainly indicates therapeutic advantage and we believe that this could be further enhanced by manipulating the dosing regimen.

To further confirm an effect on tumour size reduction using the intravenous route of administration with the polymersome formulation, Figure 11A displays the % increase in tumour weight at the end of 7 days. It was found that the tumour weight of mice in the untreated group increased to 92.3 ± 17.8% of the initial weight, whereas those in the combination free drug solution and the combination drug loaded polymersomes treated groups increased by 53.8 ± 13.0% and 40.1 ± 12.5% respectively. Furthermore, the calculation of tumour doubling time indicates that it would take 10.3 ± 1.2 days and 14.7 ± 3.7 days for the untreated group and combination free drug solution group tumours to double in size. However following treatment with the combination drug loaded polymersome formulation, tumours would take the longer time of 21.3 ± 7.9 days to double in size (Figure 11B) again confirming a negative impact on tumour growth by the polymersome formulation.

Further evaluation of systemic cardiotoxicity was carried out by the observation of the reduction in heart weights after the completion of the study (Figure 12). It was found that mice treated with combination free drug solution showed an average heart weight of 88 ± 2 mg which was lower than those of either the untreated group or the combination drug loaded polymersome treated groups which had heart weights of 120 ± 2 mg and 100 ± 1 mg respectively. Furthermore, H & E staining of hearts revealed an increase in nuclei in tissue from animals treated with the Combo Soln. compared with heart tissue from either those treated with the Combo PS or untreated animals (Figure 12 B). This suggested that cellular recruitment may be occurring to address toxic insult caused by the drugs in free solution. This was confirmed by the presence of extensive necrotic regions and evidence of cellular mobilisation in tissue harvested from animals treated with the Combo Soln. (Figure 12C) and a distinct absence of both

in animals treated with the Combo PS or untreated animal. This again demonstrates the potential benefit offered by the novel polymersome formulation and clearly demonstrates a cardio-protective effect afforded by the approach, presumably because of modifying the bioavailability of the cardiotoxic anthracycline (Dox) in the formulation.

3.6 The effect of treatment with combination polymersomes on expression of candidate gene in tumours.

RealTime ready custom plates were used to compare the expression of 40 candidate genes involved in pancreatic cancer progression. BxPC-3 tumours were harvested from control untreated tumours and tumours treated with combination free drug solutions and combination polymersomes on Day 7. Gene expression in treated groups was compared to untreated after data were normalised using a reference gene set (Beta actin, 18s ribosomal RNA and GAPDH). Expression changes for all genes are shown as scatter plots (Figure 13A). Genes were significantly different if they fell to the left of the vertical line and above or below the horizontal lines (student *t*-test). The combination solution scatter plot reveals an alteration in genetic profile compared to untreated, highlighting a significant reduction in 6 pro survival genes and a significant increase in 2 pro survival genes. The combination drug loaded polymersomes scatter plots showed a similar trend to combination free drug solution, 9 pro survival genes were down regulated and 3 pro survival genes were upregulated. However, it is notable that combination drug loaded polymersomes causes the scatter plot genes to shift to the bottom left, therefore causing a greater significant reduction in pro survival genes compared to combination free drug solutions. Figure 13B shows specific genes that are significantly up-regulated or down-regulated in either of the treatment groups compared to untreated (set at 0 % expression). Combination free drug solution and combination drug loaded polymersomes causes a reduction in the majority of genes associated with apoptosis, cancer stem cells, angiogenesis, hypoxia, poor patient survival and autophagy compared to the untreated.

The expression of three pro-apoptotic (BIK, BAX and CASP3) and one anti-apoptotic gene (BCL2) were analysed and it was unexpected that treatment with both the free and polymersome-based treatments led to down-regulation of both the pro-apoptotic

BIK and BAX genes and yet the anti-apoptotic BCL2 was also down regulated. Although the inter-relationship between these gene products is complex [58,59], it has been shown that survival of BxPC3 cells can be enhanced by upregulation of BCL2 and down-regulation of BAX [60]. Indeed, resistance to gemcitabine in this cell line has been demonstrated to result from ERK1/2 mediated up-regulation of BCL2 and down-regulation of BAX [60]. The observation that our treatments result in down-regulation of both of these genes may be fortuitous and the overall ratio of both may be resulting in a pro-apoptotic effect. The result for significant up-regulation of CASP3 following treatment with the polymersome formulation could confirm this suggestion since it is up-regulated by both intrinsic and extrinsic apoptotic signalling pathways and provides further evidence for the enhanced effects on tumour size/doubling time following treatment with the polymersome formulation.

Hypoxia is very common in pancreatic tumours and is a contributing factor in treatment failure. Hypoxic stress results in the establishment of more resistant tumours cells with an aggressive phenotype [61-64]. Pro-oncogenic RUNX2 gene becomes upregulated under hypoxic stress and is partly responsible for driving tumour progression by targeting genes associated with apoptosis, angiogenesis and metastasis [65-68]. Encouragingly, our combination free drug solution and combination drug loaded polymersome treatment causes a reduction (-78% and -99%) in RUNX2 expression compared to untreated. Furthermore, an elevated anti-angiogenic effect is noted in combination drug loaded polymersome compared to combination free drug solution via a reduction of endothelial cell recruitment and survival via VEGFC and IL-8, therefore preventing tumour vascular support [67]. Cancer stem cells (CSC) are a small population of cells that are involved in tumour initiation, growth, metastasis and resistance to therapy [69-74]. CSC are associated with pancreatic cancer progression, encouragingly, 2 of the CSC associated genes investigated (NANOG and SOX2) showed ≥10% reduction in tumours treated with the combination drug loaded polymersomes when compared with expression of those genes in tumours treated with the combination free drug solution. Our data reveal that one out of two of the autophagy marker genes (CLCN7) was down-regulated in both treatment groups. However, CLN3 was increased by 26% in combination free drug solution treatment and 233% in combination drug loaded polymersomal treatment compared to the control [75]. Autophagy is a stress response that can enhance survival of cells that

have been damaged; this is clearly playing a role after our treatment, providing survival capability for cancer cells. This suggests that targeting autophagy could provide additional therapeutic benefit and enhance overall patient survival. Furthermore, the expression of CNDP2 and CTGF [75,76] in clinical cancer biopsies were associated with poor prognosis, our findings reveal CNDP2 is elevated by both treatments. However, CTGF is increased by 145% following treatment with the combination free drug solution but interestingly significantly reduced to -87% in tumours treated with the combination drug loaded polymersome compared to untreated therefore suggesting better patient outcome. Although the above molecular genetic studies indicate positive attributes in terms of gene expression associated with treatment, particularly following treatment with the polymersome-based formulation, it may be necessary to perform these studies closer to the first treatment since tumours had clearly started to re-establish at the time of harvesting. Nevertheless, in overall terms we believe that, although a more in-depth study will be required to elucidate underlying molecular genetic mechanisms underpinning the mediation of cell death mechanism(s) elicited by the polymersome-based formulation, clear benefit in the current analysis appears to be afforded by the approach.

4.0 Conclusion

Combination cancer chemotherapy provides an important treatment tool, both as an adjuvant following the surgical removal of a tumour, and as a neoadjuvant therapy for tumours that are hard to reach and remove surgically. NP DDSs are versatile drug delivery systems capable of encapsulating large amounts of multiple drugs simultaneously, proving ideal for multi drug delivery. In addition, they offer a mechanism to combat multi drug resistant cancers and poor patient tolerance of the cytotoxic compounds utilised. Liposomes are at the forefront of NP therapy for cancer, however the problems associated with them provides the need for exploring other delivery vehicles. In this research work, we have demonstrated the formulation of electroneutral polymersomes with a hydrodynamic radius of ~132nm and PDI of ~0.43 from an amphiphilic random copolymer. The polymersomes were capable of simultaneously encapsulating three drugs namely 5-FU, Dox and LV within the aqueous core at therapeutic concentrations with an encapsulation efficiency no less

than 70% for any of the drugs incorporated. In addition, the release profile for the combined drugs was established, with each component following zero order rate kinetics for drug release within the first two hours. Polymersomes loaded with all three drugs displayed significantly better cell toxicity (36.5% cell viability) than their corresponding free drug solutions either independently (no drug generated a less than 90% cell viability) or given concomitantly (75% cell viability) with good biocompatibility determined from the blank polymersomes. The *in-vivo* behaviour of the polymersome, in terms of biodistribution, was established and found to accumulate largely in the tumour tissue with up to 6-fold accumulation in the tumour after 8 hours of injection when compared to peripheral tissues. The polymersomes encapsulating the three chemotherapeutic drugs were assessed *in-vivo* and compared with the same concentration of the three drugs in solution following both intratumoral and intravenous administration. We can report better efficacy and higher maximum tolerated dose for our combination drug loaded polymersomes when compared to the combination drug solutions after both intratumoral and intravenous administration to mice bearing ectopic BxPC3 pancreatic cancer tumours. Intratumorally injected combination drug loaded polymersomes provided a 2% decrease in tumour volume after 13 days when compared with the combination solutions which yielded a 60% increase in tumour volume following the completion of the study. A smaller 40% increase in tumour volume was observed for combination drug loaded polymersomes when given intravenously, however this was an improvement on the 53% increase displayed by tumours following treatment with the combination free drug solutions at day 7. The combination solution demonstrated extreme cardiotoxicity and weight loss in treated mice whereas only very limited toxicity was observed for the combination drug loaded polymersome. To further support these findings, the tumours were excised, and genetic profiling carried out. In addition to demonstrating gene expression profiles that were suggestive of treatment induced apoptosis, treatment with the combination drug loaded polymersomes favoured down regulation of genes that would normally drive tumour survival, suggesting decreased angiogenesis, alleviation of hypoxia-induced stress and a reduction in stem cellness. In conclusion, the results described herein using this novel formulation provide significant preclinical evidence to suggest that such formulations could play a significant clinical role in the fight against cancer. They achieve this by enabling the formulation of multiple drugs into a single, easily administered dosage form that could also preclude many of the adverse effects

associated with conventional combination drug-based approaches. In addition, they have the ability to encapsulate drugs that have both high polarities and well as those that are non-polar, thus ensuring that both drug solubility and cell permeability can be overcome from a drug delivery perspective.

Future work for these NPs will include the optimisation of payloads to ensure that the full potential of these DDSs can be met and improve upon the IV results. In addition, the introduction of drug targeting moieties can be covalently linked to the polymer backbone prior to formulation [28,46] or indeed the incorporation of activating compounds to allow for triggered release [77] further reducing unwanted side effects of these toxic therapies while reducing payload and cost. The future of simultaneous delivery using polymersomes is very promising.

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Ulster University

Statement of significance for manuscript entitled "Electroneutral polymersomes for the targeted delivery of three cancer chemotherapy drugs: In-vitro and in-vivo evaluation".

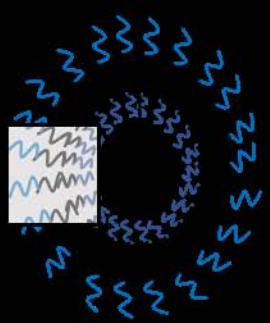
The shift in focus from mono to combination chemotherapies has led to an increased interest in the role of drug delivery systems (DDS). Liposomes, although commercialized for mono therapy, have lower loading capacities and stability than their polymeric counterpart, polymersomes. Polymersomes are growing in prevalence as their advantageous properties are better understood and exploited. Here we present a novel polymersome for the encapsulation of three anticancer compounds. This is the first time this particular polymersome has been used to encapsulate these three compounds with both an in-vitro and in-vivo evaluation carried out. This work will be of

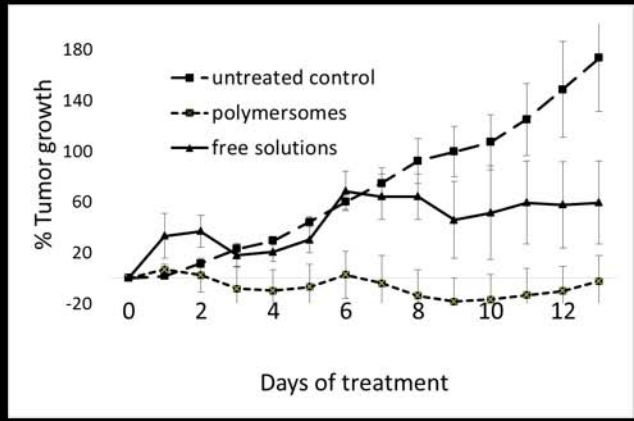
interest to those in the field of combination therapy, drug delivery, drug toxicity, multidrug resistance, liposomes, DDS and polymersomes.

Tables

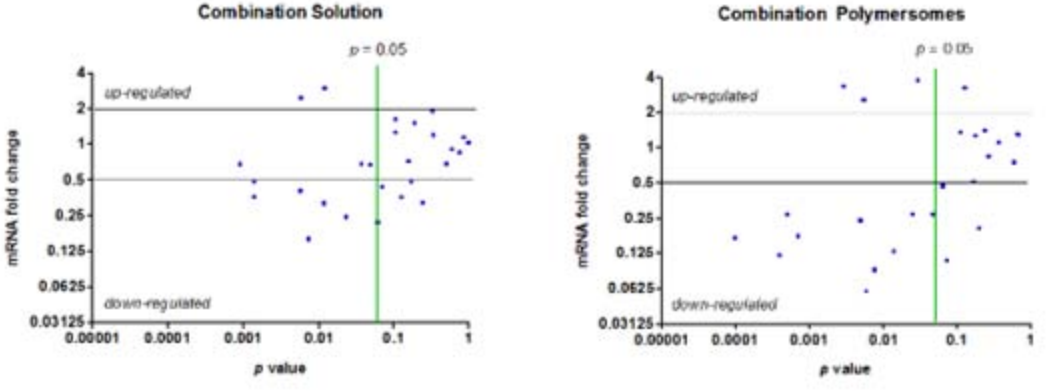
Table 1. Characterisation and encapsulation efficiency of various polymersomal formulations, both with single occupancy drugs and combination occupancy.

	Blank	**DOX only**	**5-FU only**	**LV only**	**Combination of all three**
Size (nm)	141 ± 18.2	235.6±35.5	205 ± 3.3	175 ± 38.2	133 ± 35.5
PDI	0.27 ± 0.06	0.40±0.01	0.44 ± 0.02	0.4 ± 0.05	0.43 ± 0.14
Zeta (mV)	-0.82 mV ±0.2	-1.15±1.11	-1.02±0.93	-1.6±0.6	-2.79 ±1.22
Encapsulation efficiency (%)	N/A	87.2±1.7	81.4±1.6	89.7±0.5	**Dox** 81.5 ± 5.5 **5-FU** 74.5 ± 20.1 **LV** 72.1 ± 0.7

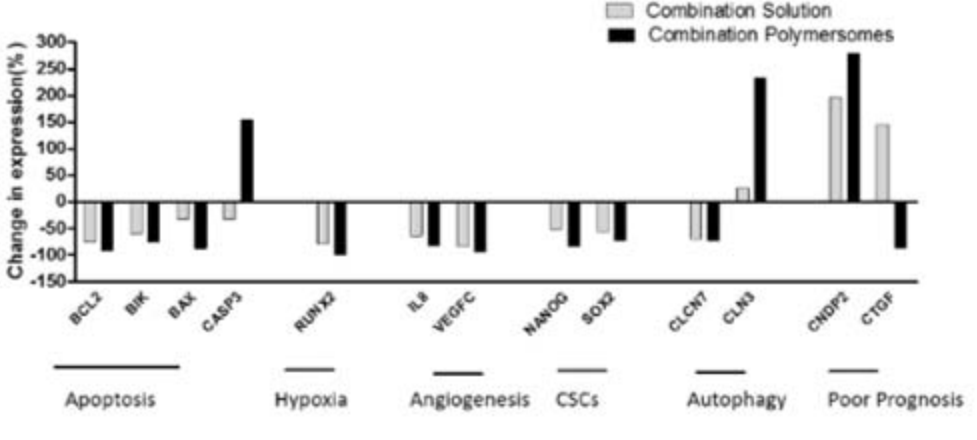


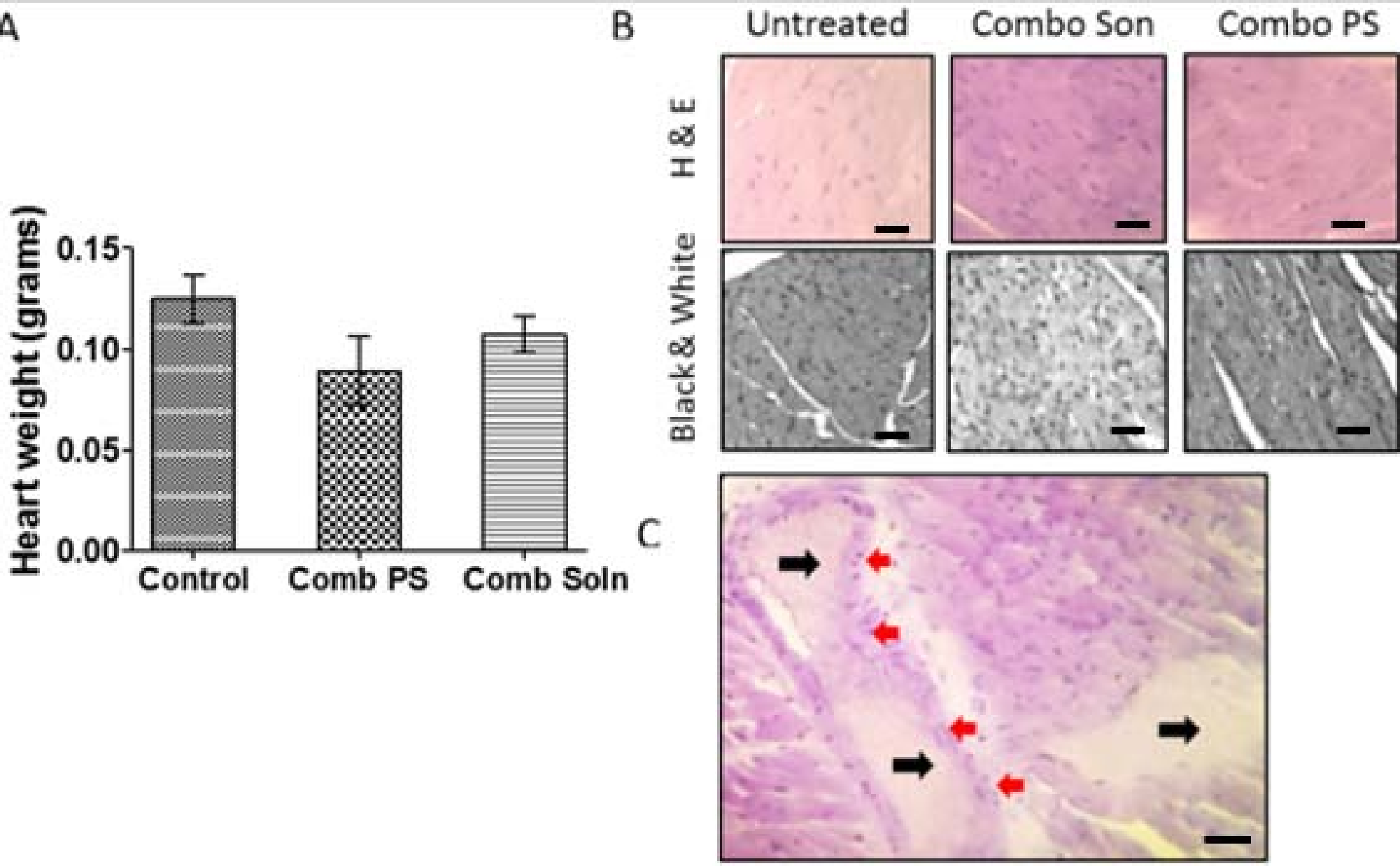

180
140
100
60
20
-20
% Tumor growth
untreated control
polymersomes
free solutions
0
2
4
6
8
10
12
Days of treatment

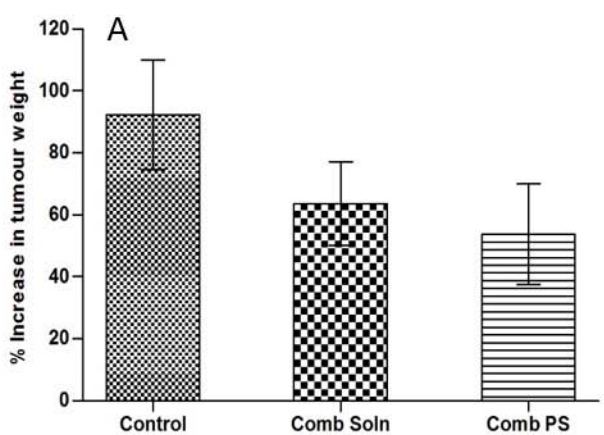
(A)

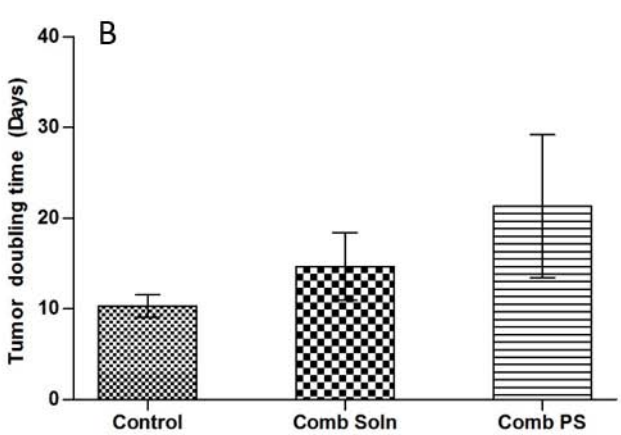

Combination Solution
p = 0.05
up-regulated
down-regulated
mRNA fold change
4
2
1
0.5
0.25
0.125
0.0625
0.03125
0.00001
0.0001
0.001
0.01
0.1
1
p value
Combination Polymersomes
p = 0.05
up-regulated
down-regulated
mRNA fold change
p value

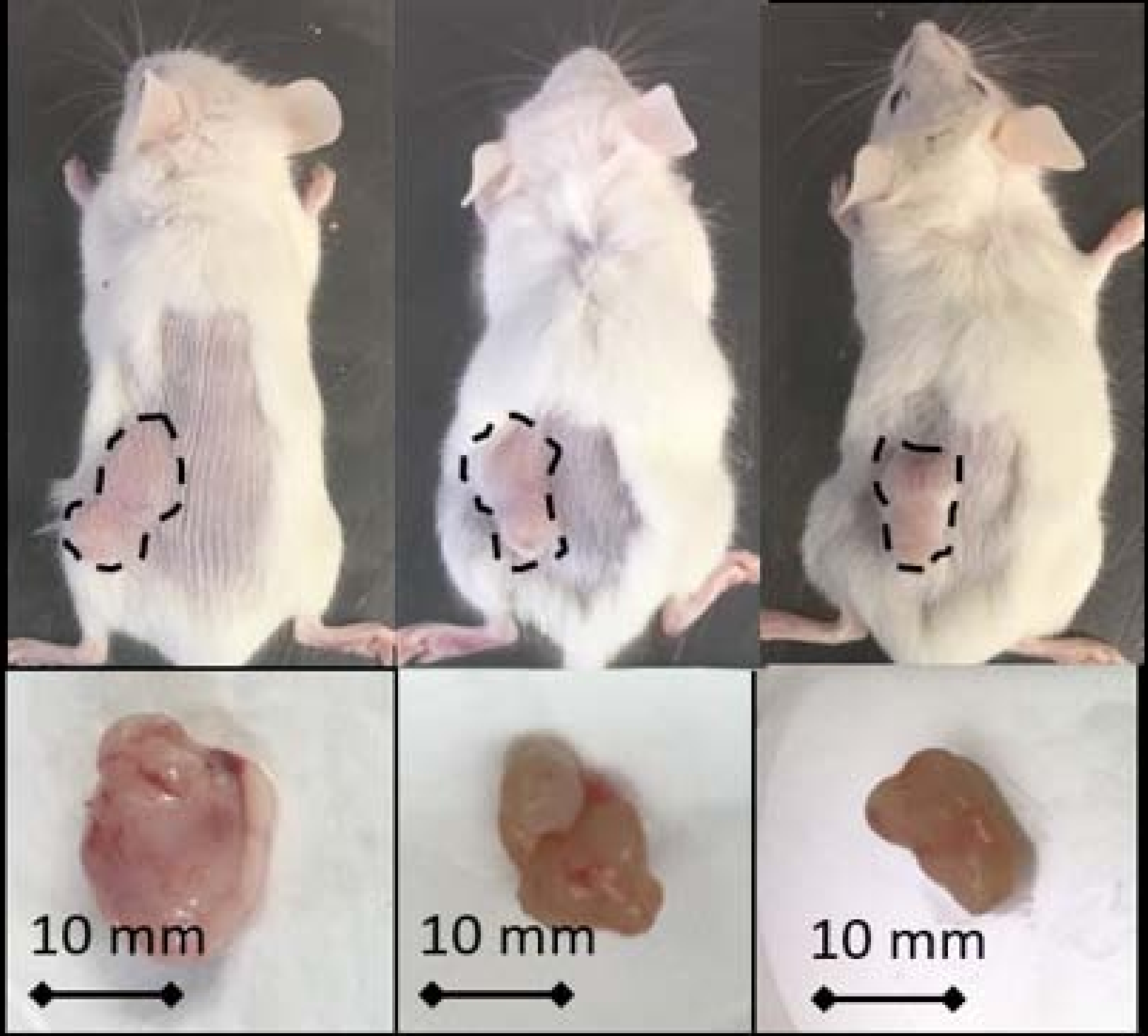
(B)

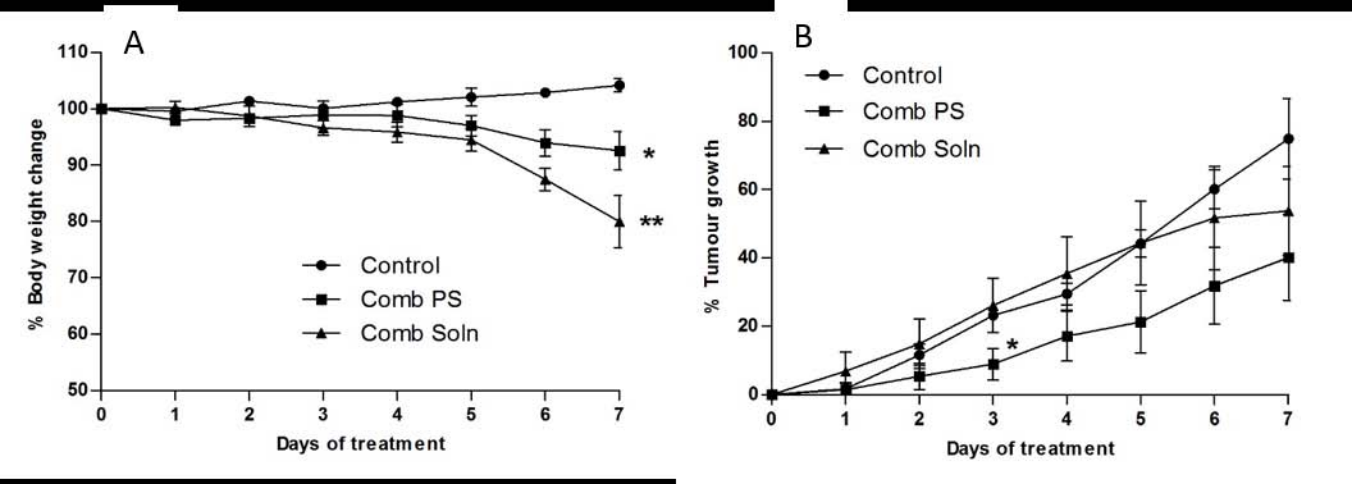

Combination Solution
Combination Polymersomes
Change in expression(%)
300
250
200
150
100
50
0
-50
-100
-150
BCL2
BIK
BAX
CASP3
RUNX2
IL8
VEGFC
NANOG
SOX2
CLCN7
CLN3
CNDP2
CTGF
Apoptosis
Hypoxia
Angiogenesis
CSCs
Autophagy
Poor Prognosis

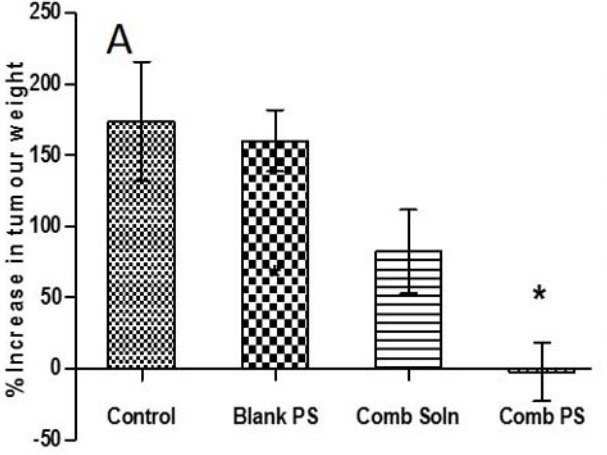

A
Heart weight (grams)
0.15
0.10
0.05
0.00
Control
Comb PS
Comb Soln
B
Untreated
Combo Son
Combo PS
H & E
Black & White
C

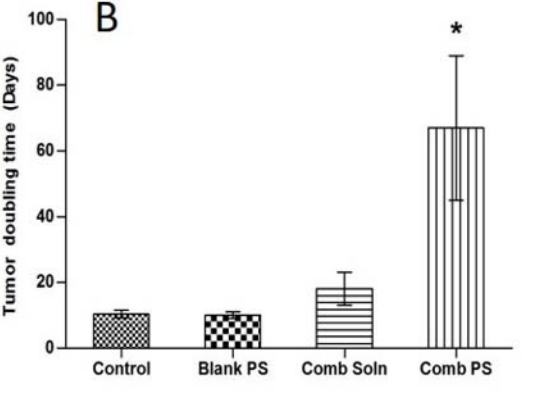

A
% Increase in tumour weight
120
100
80
60
40
20
0
Control
Comb Soln
Comb PS

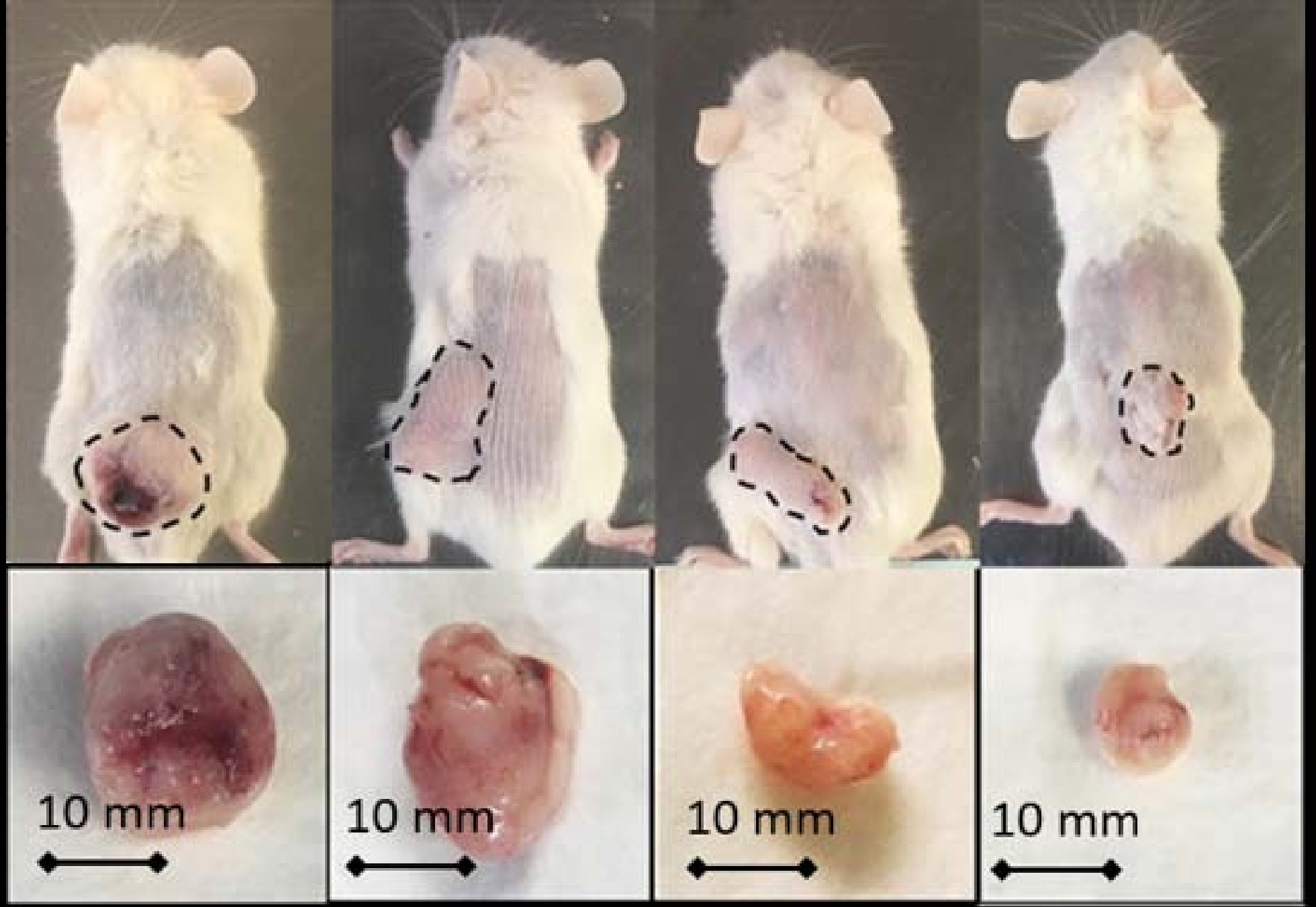

B
Tumor doubling time (Days)
40
30
20
10
0
Control
Comb Soln
Comb PS

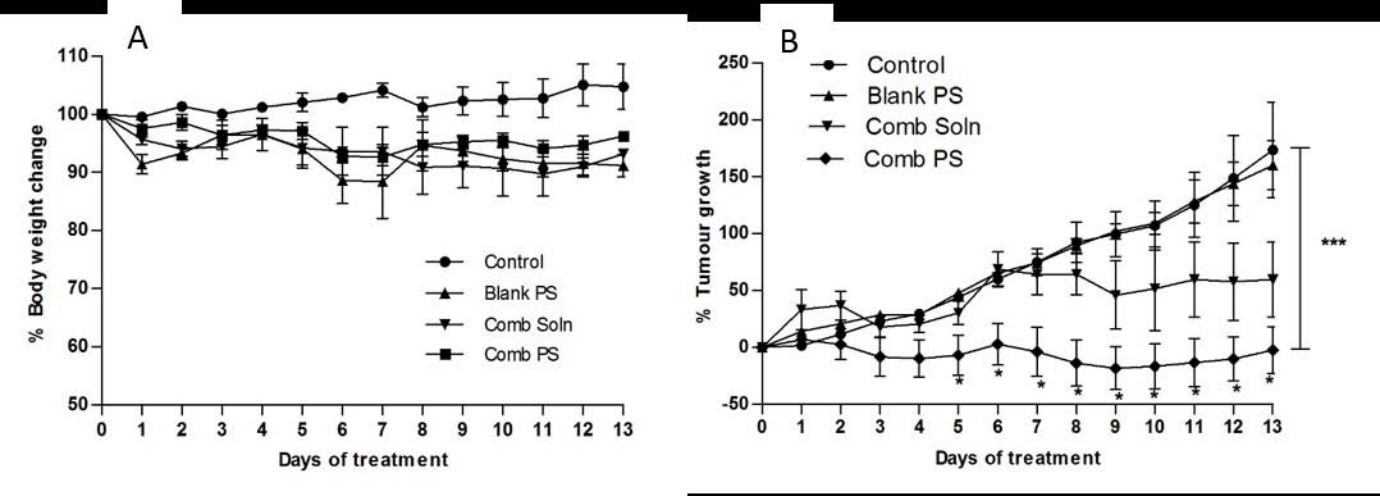

10 mm
10 mm
10 mm

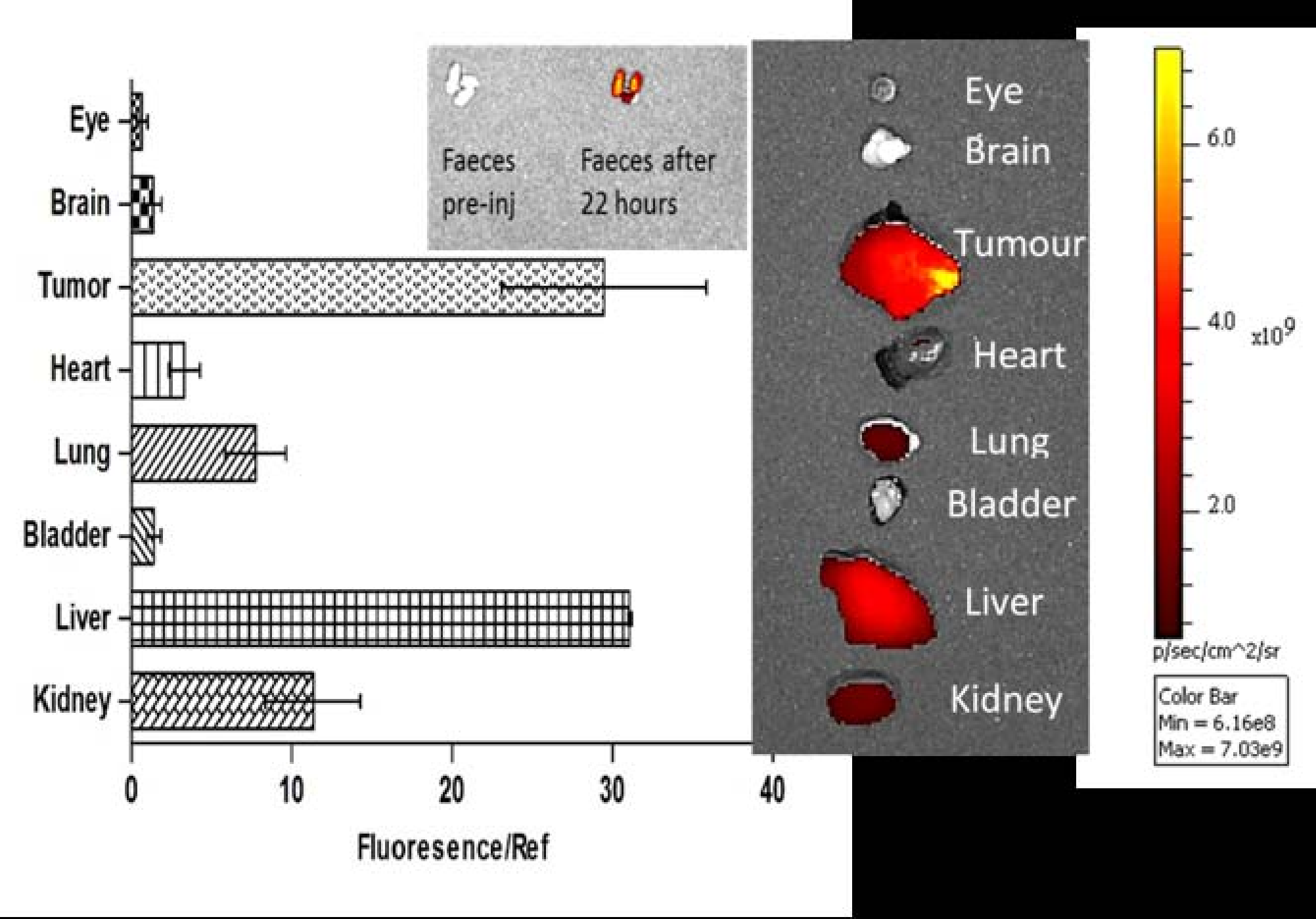

A
% Body weight change
Days of treatment
Control
Comb PS
Comb Soln
*
**
B
% Tumour growth
Days of treatment
Control
Comb PS
Comb Soln
*


A
% Increase in tumour weight
250
200
150
100
50
0
-50
*
Control
Blank PS
Comb Soln
Comb PS

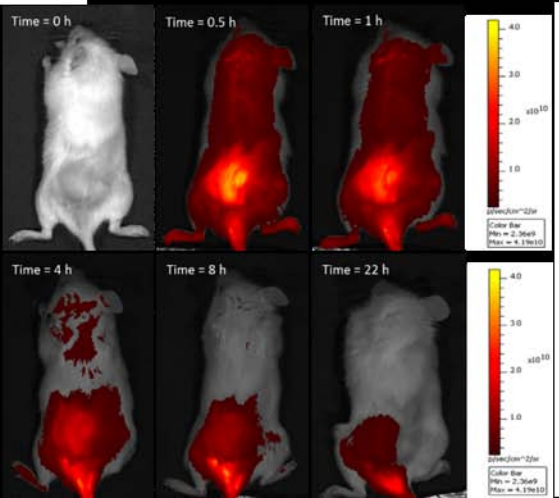

B
Tumor doubling time (Days)
100
80
60
40
20
0
*
Control
Blank PS
Comb Soln
Comb PS


10 mm
10 mm
10 mm
10 mm

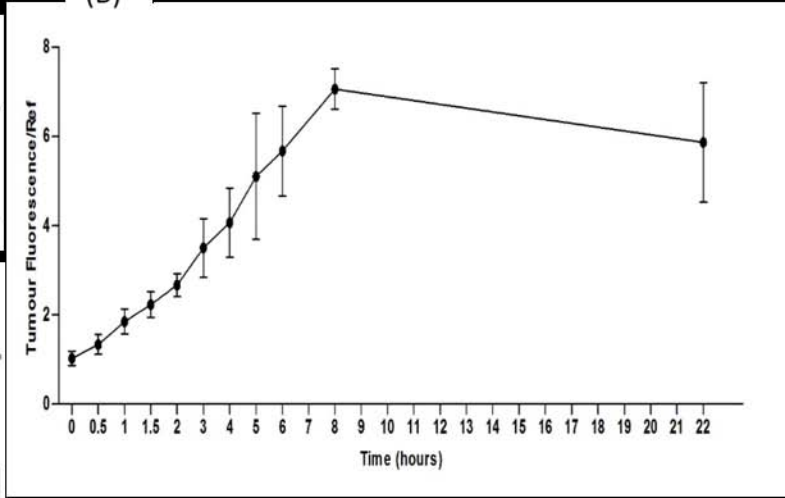

A
% Body weight change
110
100
90
80
70
60
50
0 1 2 3 4 5 6 7 8 9 10 11 12 13
Days of treatment
Control
Blank PS
Comb Soln
Comb PS
B
% Tumour growth
250
200
150
100
50
0
-50
0 1 2 3 4 5 6 7 8 9 10 11 12 13
Days of treatment
Control
Blank PS
Comb Soln
Comb PS
***

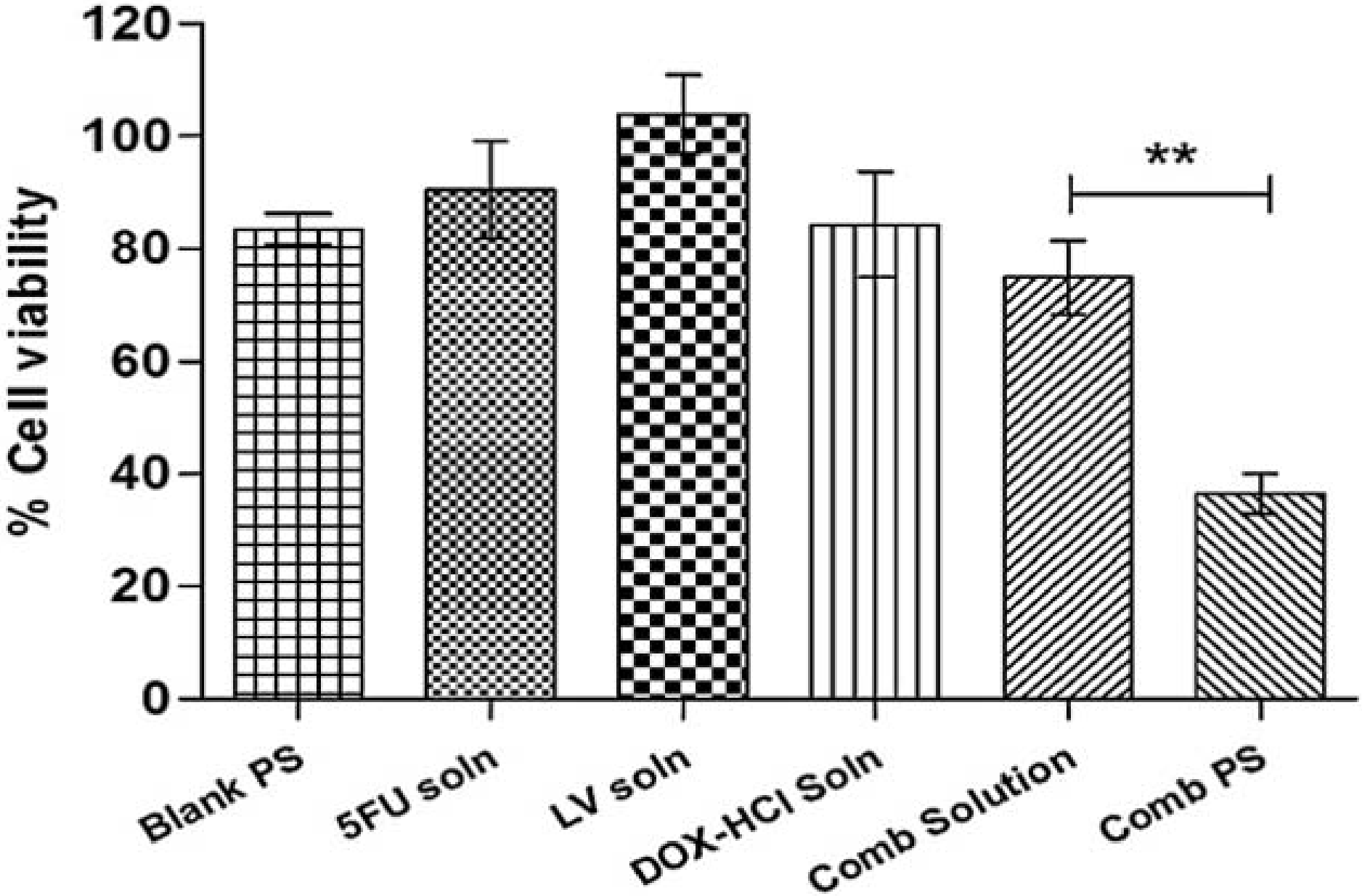

Eye
Brain
Tumor
Heart
Lung
Bladder
Liver
Kidney
0
10
20
30
40
Fluoresence/Ref
Faeces pre-inj
Faeces after 22 hours
Eye
Brain
Tumour
Heart
Lung
Bladder
Liver
Kidney
6.0
4.0
2.0
x10^9
p/sec/cm^2/sr
Color Bar
Min = 6.16e8
Max = 7.03e9

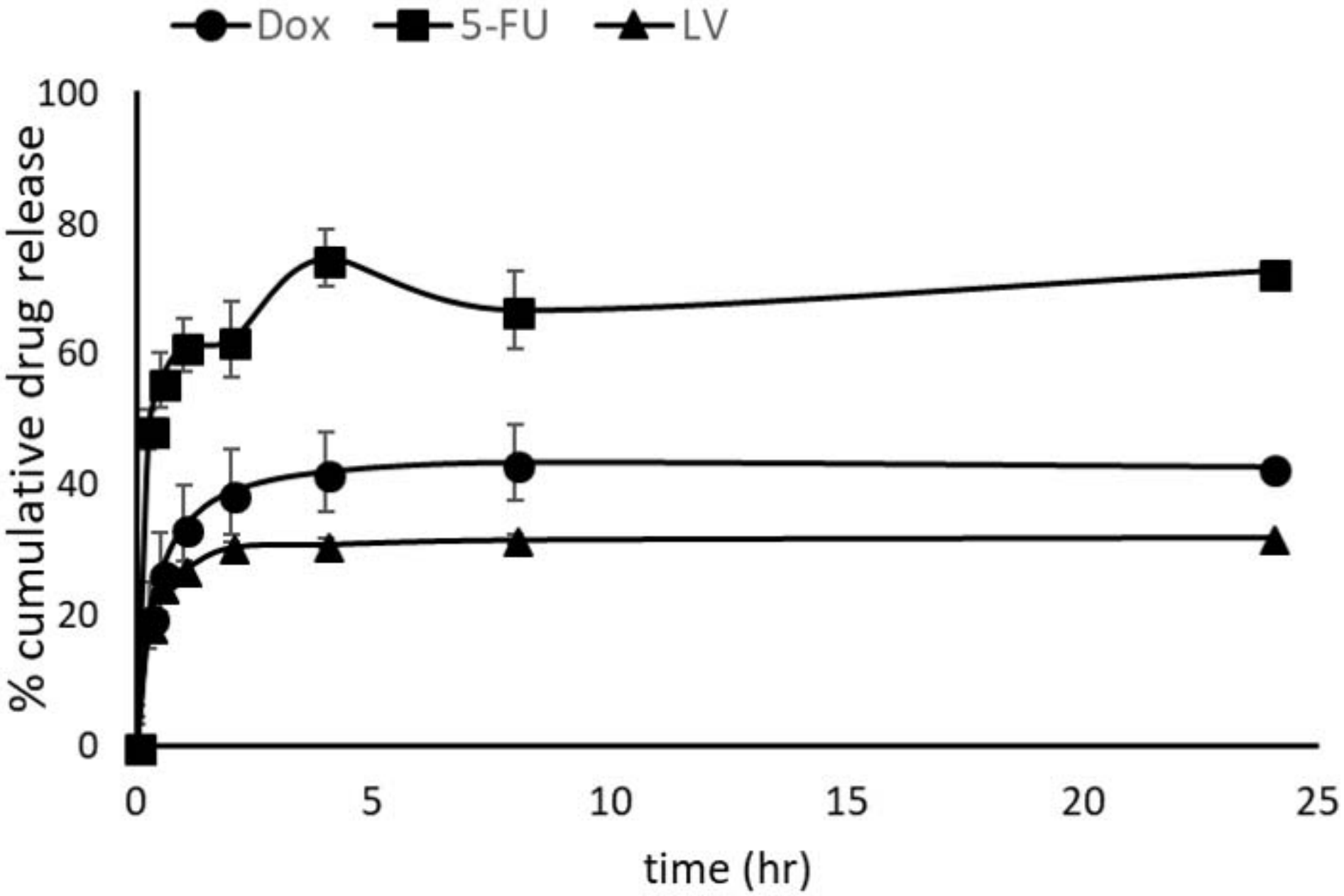
(A)

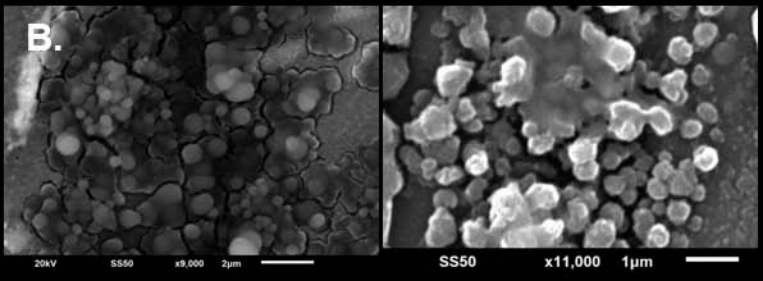

Time = 0 h
Time = 0.5 h
Time = 1 h
Time = 4 h
Time = 8 h
Time = 22 h
Color Bar

(B)


Tumour Fluorescence/Ref
Time (hours)
0 0.5 1 1.5 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22


120
100
80
60
40
20
0
% Cell viability
**
Blank PS
5FU soln
LV soln
DOX-HCl Soln
Comb Solution
Comb PS


Dox
5-FU
LV
100
80
60
40
20
0
% cumulative drug release
0
5
10
15
20
25
time (hr)


B.
20kV SS50 x9,000 2µm
SS50 x11,000 1µm